The low energy emitting states of the Lhca4 subunit of higher plant photosystem I

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Received 20 December 2004; revised 8 February 2005; accepted 21 February 2005

Available online 10 March 2005

Edited by Peter Brzezinski

Abstract The selectively red excited emission spectrum, at room temperature, of the in vitro reconstituted Lhca4, has a pronounced non-equilibrium distribution, leading to enhanced emission from the directly excited low-energy pigments. Two different emitting forms (or states), with maximal emission at 713 and 735 nm (F713 and F735) and unusual spectral properties, have been identified. Both high-energy states are populated when selective excitation is into the F735 state and the fluorescence anisotropy spectrum attains the value of 0.3 in the wavelength region where both emission states are present. This indicates that the two states are on the same Lhca4 complex and have transition dipoles with similar orientation.

Keywords: Anisotropy spectrum; Dipole orientation; Fluorescence bandshape; Lhca4; Low energy chlorophyll forms

1. Introduction

Photosystem I of higher plants is a multi-subunit membrane pigment–protein complex, composed of two moieties: the central chlorophyll *a* binding core complex and the peripheral antenna. The peripheral antenna consists of four different LHCI (Lhca 1–4) chlorophyll *a*/*b* binding proteins [1,2]. The structure of PSI-LHCI suggests that Lhca polypeptides are bound at one side of the core complex [3,4] in the form of two heterodimers. According to biochemical data, the purified Lhca polypeptide binds, on average, 10 chl molecules [5,6], and are organized in five dimers, giving a total of 80–100 chlorophyll *a* + *b* molecules and 40 xanthophylls bound to LHCI. However, the recent crystal structure of plant PSI-LHCI complex indicate that two Lhca dimers are present with 14–15 chl molecules per polypeptide, giving a total of 55–60 chlorophyll *a* + *b* molecules, including "gap" and "connecting" Chls, at the interface between protein subunits [4]. It should be noted that changes in Lhca polypeptide levels in plants grown at different irradiances have been observed [7].

An intriguing property of intact PSI is the presence of a significant amount of oscillator strength in its low energy absorption tail, due to the presence of red spectral chlorophyll *a* forms, or states (e.g. [8]). Their combined Q_y oscillator strength is approximately equivalent to one twentieth of the total Q_y oscillator strength in higher plants [9] and most of these forms seem to be associated with the LHCI complexes [10]. At equilibrium and at room temperature about 80% of the excited states population is on these low energy molecules and energy is transferred to the high energy states through a relatively slow thermally activated process [11]. Evidence from cyanobacteria [12–14] and Lhca3–4 [15,16] indicate that these low energy chlorophyll states are generated by intense coulombic interactions between the transition dipoles of chlorophyll dimers, mediated by the protein ligands [16] and a pair of chlorophyll a molecules on both Lhca3 and Lhca4 have been proposed to be involved in long wavelength absorption [4,16,17]. It was proposed that the long wavelength fluorescing state, F730, is a property of Lhca3 and Lhca4 [16,18–21]. The main biological function of these red states is that of increasing the light harvesting by leaves exposed to a light environment enriched in wavelengths above 690 nm, due to shading by other leaves [22].

An analysis of the long wavelength fluorescence of a LHCI preparation as a function of temperature [10] has suggested the presence of multiple red forms, characterized by an optical reorganization energy greater than that for the bulk pigments absorbing at wavelengths around 680 nm, due to a stronger coupling of the electronic transitions to phonon modes [12,23,24]. Recently, a direct determination of the fluorescence bandshape of the F735 long-wavelength chlorophyll state, at room temperature, has been obtained by means of selective excitation within its absorption band [25,26]. This emission band has the broadest FWHM ever reported for a Q_y chlorophyll a fluorescence; its value is around 55 nm (at 280 K) whereas solution values are around 20 nm and non-red-shifted antenna chlorophylls around 10–12 nm (e.g. [27]) at comparable temperatures.

The selectively red excited LHCI room temperature emission spectra recently analyzed [26] has led to the conclusion that the

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Abbreviations: FWHM, full width at half maximum; Lhca1–4, individual polypeptides composing the external antenna of photosystem I; PSI, photosystem I; P700, photosystem I primary electron donor; LHCI, light harvesting complex I

0014-5793/$30.00
doi:10.1016/j.febslet.2005.02.057

long wavelength emission of isolated LHCI is composed of two contributions, with maxima at around 713 and 735 nm, which were suggested to be localized on the same LHCI complex. Moreover, on the basis of the results obtained with in vitro reconstituted LHCI monomers [6,28], it was demonstrated that F735 is mainly associated with Lhca4. In this paper, the in vitro reconstituted complex Lhca4 has been directly analyzed, measuring the room temperature pre-equilibrium fluorescence of the low-energy states upon direct excitation.

2. Materials and methods

2.1. DNA constructions and isolation of overexpressed Lhca apoproteins from bacteria

cDNAs of Lhca4 from *Arabidopsis thaliana* were expressed in SG13009 strain of *E. coli* [6]. Apoproteins were isolated from the SG13009 strain of *E. coli* transformed with these constructs following a protocol previously described [29,30].

2.2. Reconstitution and purification of protein–pigment complexes

Reconstitution were performed as described in [31] with the following modifications: the reconstitution mixture contained 420 μg of apoprotein, 240 μg of chlorophylls and 60 μg of carotenoids in total 1.1 ml. The Chl *a*/*b* ratio of the pigment mixture was 4.0. The pigments used were purified from spinach thylakoids.

2.3. Protein and pigment concentration

HPLC analysis was as in [32]. Chlorophyll to carotenoid ratio and Chl *a*/*b* ratio was independently measured by fitting the spectrum of acetone extracts with the spectra of individual purified pigments in acetone 80% [33].

2.4. Fluorescence measurements

Samples were resuspended in a buffer containing tricine (10 mM, pH 7.8) and DM (0.015%) to an OD of about 0.3/cm at the absorption maximum. These buffer conditions ensures sample stability, as previously observed for LHCI [26]. No changes to the emission bandshape were observed on lowering sample concentration further on (not shown). Steady state fluorescence emission spectra were measured, at 8 °C, with a 3 mm path-length cuvette, using a 90° geometry setup, through a 300 cm focal length imaging spectrograph (SpectraPRo-300i, Acton Research Co. USA) equipped with a 300 lines grating, connected to a liquid nitrogen cooled CCD array detector (Princeton Applied Research) with ST-138 Camera Controller (Princeton Applied Research). The spectral resolution of this setup is 0.28 nm. Measured fluorescence spectra were corrected for the instrument wavelength sensitivity. The excitation beam was provided by the water-cooled xenon lamp and monochromator of a J-500 spectropolarimeter (Jasco). The transmittance half width was adjusted to the minimum that gives a detectable fluorescence signal. Fluorescence spectra obtained with excitation into the Q_y absorption band were corrected for the excitation spike by means of the measured spectrum of scattered light of a glycogen suspension. In this way it was possible to completely eliminate the scattering spike in the emission spectra to within less than ±5 nm of the spike maximum for all excitation wavelengths. Fluorescence anisotropy measurements were performed using the same experimental setup by the usual methods (e.g. [34]), with polarizers selecting the vertical component for the excitation beam and both the vertical and horizontal components for emission and calculating the anisotropy factor $r = \left(\frac{I_{\parallel}}{I_{\perp}} - 1\right) / \left(\frac{I_{\parallel}}{I_{\perp}} + 2\right)$. The excitation wavelength used was 730 nm. Detection polarization bias was corrected for by using horizontally polarized excitation [34].

3. Results and discussion

The room temperature emission spectra of an in vitro reconstituted Lhca4 preparation were measured by non-selective excitation at 500 nm and also in the range 707–740 nm, where excitation is selectively into the low energy pigment state(s). In Fig. 1, the Lhca4 emission spectrum obtained with non-selective excitation at 500 nm is compared with the emission spectra measured with excitation in the red tail of the Lhca4 absorption spectrum at 720, 730 and 740 nm. The spectra are normalized at 685 nm where the bulk emission is maximal. This seems to be an adequate normalization procedure as it can be seen that, in this way, all spectra become normalized over the entire bulk pigment emission region (Fig. 1). The emission spectrum excited at 500 nm, a wavelength that excites non-selectively all the pigments types present in an Lhca4 preparation, is taken as representative of the emission spectrum due to a thermally equilibrated excited state distribution, as already shown for the native LHCI preparation [26]. It is clear that, as is the case for LHCI and PSI-LHCI [25,26], excitation into the red absorption tail of the Lhca4 complex leads to a non-equilibrium emission distribution, with a marked enhancement of the red emission compared to the "bulk" emission peak at around 685 nm. Interestingly, non-equilibrium steady state fluorescence spectra have also been recently measured in a PSI preparation from *Thermosynechococcus elongatus* core excited selectively in the red chlorophyll spectral region [35], indicating that this is a property of chlorophyll arrays containing red forms. As previously shown [25,26], the emission spectrum can be represented as a linear combination of emission bandshapes of the different states contributing to the overall emission distribution, weighted by the respective excited state population terms. These weighting terms are consistent with the equilibrium distribution, when an excited state equilibrium distribution is reached before emission, or are distributed according to the excited transfer dynamics proper of the systems. Thus, in general terms, an emission spectrum, due to n different emission sources with emission spectrum $E_n(\lambda)$, can be written as $\mathrm{PE}(\lambda) = \sum_n P_n E_n(\lambda) + \sum_n P_n^* E_n(\lambda)$, where the first term represent the emission spectrum at equilibrium and the second term is the emission from the non-relaxed

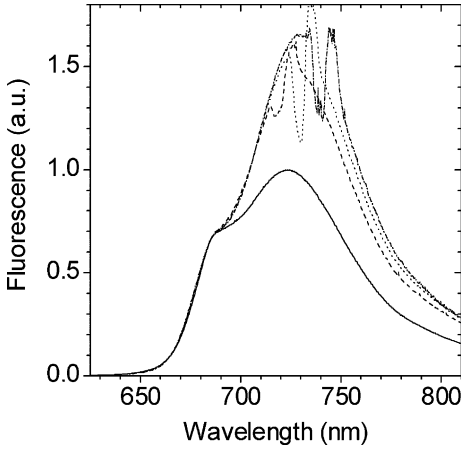

Fig. 1. Steady state emission spectra of the in vitro reconstituted Lhca4. Spectra have been measured with excitation at 500 (solid), 720 (dash), 730 (dot) and 740 nm (dash-dot). All spectra are normalized to the bulk antenna emission around 685 nm.

excited states, integrated over their excited state lifetime. As the pre-equilibrium emission is intense upon low energy excitation (Fig. 1), it is expected that equilibration of this state(s) is slow. This was previously confirmed by the slow red state-bulk spectral evolution measured in the native LHCI preparation [26] and which has also been confirmed in the present Lhca4 sample (unpublished data). This latter sample has a 2.4 ns DAS component with dominant amplitude which is markedly red shifted with respect to the faster (950 and 170 ps) DAS. Inspection of the chlorophyll positions in the crystallographic structure of PSI-LHCI [4] gives no clue as to reasons for this slow equilibration (unpublished data). However, it was previously noticed in PSI-LHCI that the low energy donor states transfer to acceptor states which are about 4 kT higher in energy at room temperatures [11]. This yields a Boltzmann factor of about 50 which, when combined with the 3.6 ps component for bulk-red form transfer observed by Gobets et al. [36] for native LHCI, suggests a slow uphill energy transfer with an apparent lifetime of the order of several hundreds of picoseconds. This would then explain the large pre-equilibrium fluorescence observed when excitation is into the low energy band.

When the emission spectrum at equilibrium, $E(\lambda)$, is known, the difference after normalization, $\Delta E(\lambda) = \mathrm{PE}(\lambda) - E(\lambda)$, yields the bandshape of the pre-equilibrium distribution. In the case where only a single spectral form absorbs at the wavelength of the excitation beam, the difference spectrum is the emission bandshape of this state. This is the case for measurement with LHCI [26] where the bandshape of the F735 state was obtained. The same approach has now been used here for the Lhca4 emission spectra (Fig. 1).

The difference spectra $\Delta E(\lambda)$, when the pre-equilibrium emission spectra after selective excitation of the F735 state at 720, 730 and 740 nm are used, are shown in Fig. 2A. In Fig. 2B the $\Delta E(\lambda)$ spectra obtained using selective excited emission spectra at 707, 710 and, again, 740 nm are presented. The spectral distribution shown in Fig. 2A have only minor differences on the high energy side and have the same bandshape characteristics already found for native LHCI [26]: an unusually wide bandwidth, when compared to the emission spectrum of bulk chlorophyll forms (e.g. [37]), a marked asymmetry towards the low energy side and a maximum that can be estimated around 735 nm. This bandshape is interpreted as the emission bandshape of the redmost energy state(s) of Lhca4. Excitation at the two shorter wavelengths gives $\Delta E(\lambda)$ spectra broader towards the high energy side, with blue shifted maxima (Fig. 2B). This blue shifting of the difference emission spectra at the higher excitation energy indicates that another state(s) with a less red-shifted emission contribute to these difference spectra, as already observed for LHCI [26].

To gain further insight into the different emission contributions composing the Lhca4 emission spectrum, some emission curve fitting analyses are presented in Fig. 3. The red emission tail of the measured Lhca4 emission spectrum is due to a main red-form(s) emission contribution plus a small contribution due to the vibronic tail of the bulk-chlorophyll emission. In Fig. 3A, the low energy bandshape obtained by excitation at 740 nm has then been used to describe the red tail of the room temperature equilibrated emission of Lhca4, in addition to a small contribution (~10%) due to the vibronic emission contribution of the bulk pigments. To this end, the F735 bandshape has been normalized to give a 90% contribution to the extreme red tail of the overall emission spectrum. The remaining emission distribution, obtained after subtraction of the F735 bandshape (Fig. 3A), shows a "bulk" emission spectrum with marked asymmetry towards low energy, as already seen for the native LHCI preparation [26]. This indicates the presence of, at least, another long wavelength emission form, blue shifted with respect to the F735 state. This result is independent of the F735 normalization factor chosen (see above). It is interesting to note that an asymmetric emission spectrum was obtained measuring the fluorescence emission, at 77 K, of an Lhca4 preparation that, after mutation in the ligand of the chlorophyll molecules proposed as a source of the red-forms, is depleted of the long wavelength emission contribution [15]. The additional emission was proposed to be around 702 nm, the same wavelength associated with a low temperature structure in the fluorescence spectrum of native LHCI [24] but associated to an emitting form which was suggested to be located on the Lhca2–3 dimer. To obtain the bandshape of this blue-shifted emission contribution, we have compared the $\Delta E(\lambda)$ spectra obtained using the selectively excited emission spectra at 740 and 707, 710 nm (Fig. 2B)

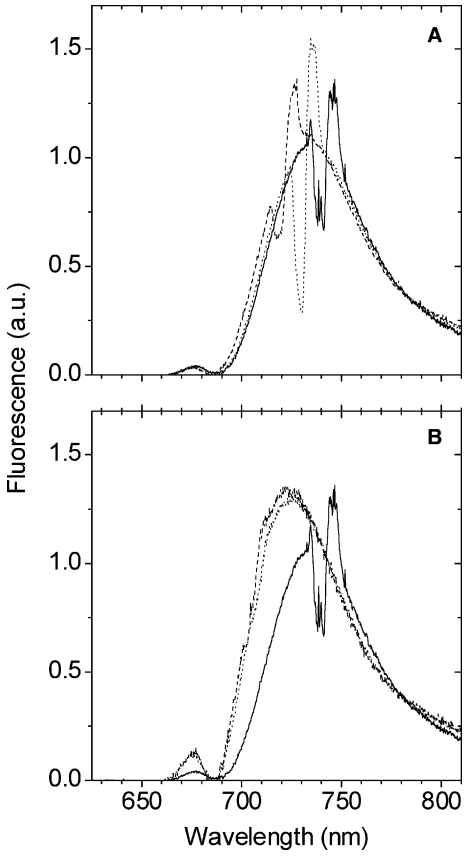

Fig. 2. Difference emission spectra $\Delta E(\lambda) = \mathrm{PE}(\lambda)|_{\lambda ex} - E(\lambda)$. $E(\lambda)$ is the steady state emission spectrum measured with excitation at 500 nm; $\mathrm{PE}(\lambda)|_{\lambda ex}$ are the steady state emission spectra measured with excitation at 720 (dash), 730 (dot) and 740 nm (solid) (A) or at 707 (dash), 710 (dot) and, again, 740 nm (solid) (B). Each difference spectrum $\mathrm{PE}(\lambda)|_{\lambda ex}$ is obtained after normalization of the $E(\lambda)$ spectrum to the respective $\mathrm{PE}(\lambda)$ spectrum at 685 nm. The spectra are shown after normalization to the long-wavelength tail.

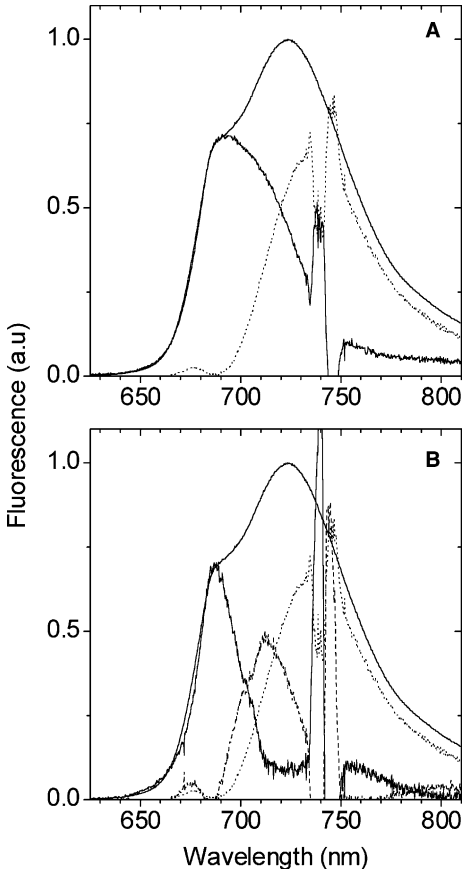

Fig. 3. Lhca4 steady state emission spectrum $E(\lambda)$ with excitation at 500 nm. (A) Within the $E(\lambda)$ spectrum the F735 (dot) for a weighting factor of 0.9 and the difference $E(\lambda) - \text{F735}$ (solid) are shown. (B) Within the $E(\lambda)$ spectrum the difference $\Delta E(\lambda)|_{707,\ 710} - \Delta E(\lambda)|_{740}$ is shown (dash). This difference spectrum defines the F713 emission band of Lhca4. Also shown (solid) is the difference spectrum $(E(\lambda) - \text{F735}) - \text{F713}$. The intensity of the sub-bands are scaled to reconstruct the $E(\lambda)$ spectrum.

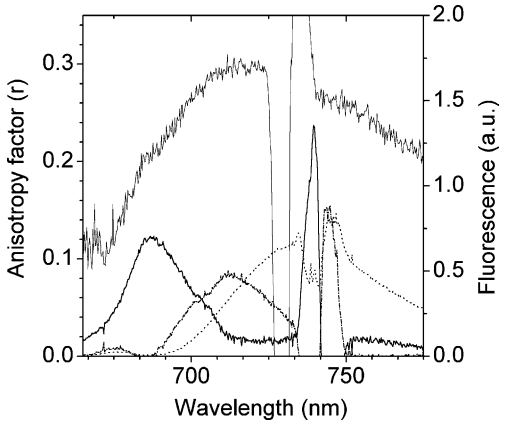

Fig. 4. Fluorescence anisotropy spectrum of Lhca4. The excitation wavelength is 730 nm. For further details see Section 2. The different sub-bands comprising the steady state fluorescence emission spectrum, as shown in Fig. 3B, are also shown, with the aim to directly envisage the sub-band contribution over the wavelength range analyzed.

where a blue shifted contribution is present. The bandshape of the blue shifted contribution has been obtained as a difference between the bandshape $\Delta E(\lambda)|_{<720}$, calculated with the emission spectra measured with excitation at 707 and 710 nm, and the bandshape $\Delta E(\lambda)|_{=740}$, obtained with excitation at 740 nm, after normalization to the red tail area below 760 nm. Two very similar bandshapes are obtained (not shown) and their sum is shown in Fig. 3B. This bluer bandshape, peaking around 713 nm, has been subtracted from the "bulk" emission of Fig. 3A, after a normalization that allowed a red vibronic contribution (~20%) to the F685 emission spectrum. In Fig. 3B the Lhca4 equilibrium emission spectrum is shown with the contribution due to the "bulk", and to the two red emission spectra. The spectral properties of the F713 contribution are similar to those obtained with LHCI [26] with a bandwidth of around 27 nm. This conclusively demonstrates that the Lhca4 monomer binds two distinct red forms, emitting at 713 and 735 nm, with different bandshapes. The F713 form seems to be associated with the same Lhca4 complexes as the F735 state, as it is populated when excitation is selectively into the F735 state, i.e., at 740 nm. In a recent work [26] on LHCI, using fluorescence polarization measurements we suggested that the F713 emitting state was associated with the same LHCI complex, probably Lhca4, as the F735 one. This suggestion is now directly substantiated by the data shown in Fig. 4 in which fluorescence anisotropy measurements with excitation at 730 nm, well inside the absorption region of the F735 red emission form, have been performed by measuring the polarized fluorescence over the entire emission interval. The anisotropy increases across the bulk chlorophyll band to reach a maximum value of about 0.3 around 705 nm, then remains constant for about 20 nm and, probably, inside the spike region begins to decrease, in general accordance with the data obtained with LHCI [26]. As is the case for LHCI, the maximum anisotropy value is reached in the wavelength interval were also the emission from the F713 state is present (Fig. 4) indicating that the two states giving rise to emission at 735 and 713 nm are connected via energy transfer and that have similar orientation of the emission transition dipoles. It is interesting to note that the presence of two different red emitting states has been suggested in reconstituted Lhca2 subunit, but the two different forms are associated with different subsets of the protein ensemble [38].

To summarize, using a reconstituted Lhca4 preparation to measure fluorescence emission by non-selective and red-selective excitation we directly demonstrated that at least two different red emission states, F713 and F735, are present on the same Lhca4 complexes. Moreover, these two states have emission transition dipoles with similar orientation.

Previous work with Lhca 3 and Lhca4 has shown that the red-shifted absorption forms yielding the 735 nm emission are originated from an excitonic interaction between two Chl *a* molecules located, respectively, in binding sites A5 and B5 within the Lhc structure [16,17]. Further analyses with Lhca2, which exhibits a 702 nm lowest energy emission, were interpreted in term of the same Chl *a* chromophores bound to sites A5 and B5 involved in an excitonic interaction which yields a lower amplitude red-shift owing to a larger inter-chromophore distance. This was attributed to the

substitution of the Asp residue coordinating Chl A5 to a more bulky His, thus bringing the two chromophores apart [16,38]. A difference in Mg–Mg separation between the A4 and B5 chromophores in Lhca4 (~7.8 Å) with respect to Lhca2 (~10.5 Å) has been observed in the recent crystal structure of plant PSI [4,39]. However, the Mg–Mg distance between the same chromophores is about 9.3 Å in Lhca3 and 8.3 Å in Lhca1, which is not in direct correlation with the presence of red forms.

The present finding of a 713 nm emission from recombinant Lhca4 raises the question of the structural basis of this state. At the present moment we are unable to draw any specific conclusions on this. However it should be noted that in the PSI crystal structure [4,39] a number of chromophore couples, in addition to the A5–B5, have Mg–Mg separation in the range 7–10 Å (A1–B1, A2–B2, A3–B3, A6–B6) and these are present in all Lhca complexes. Moreover it should be noted that, in all antenna complexes, a third chromophore, B1, has a Mg–Mg separation of about 10 Å with respect to B5. A Chla trimer in the Psab peripheral antenna of PSI of *Synechococcus elongatus* [40] has been proposed as a putative candidate for bathochromically shifted chlorophylls.

Acknowledgement: This work was partially financed by the Grant FIRB RBAU01E3CX.

References

[1] Mullet, J.E., Burke, J.J. and Arntzen, C.J. (1980) Chlorophyll proteins of photosystem I. Plant Physiol. 65, 814–822.
[2] Bassi, R. and Simpson, D. (1987) Chlorophyll–protein complexes of barley photosystem I. Eur. J. Biochem. 163, 221–230.
[3] Boekema, E.J., Jensen, P.E., Schlodder, E.J., van Breemen, F.L., van Roon, H., Scheller, H.V. and Dekker, J.P. (2001) Green plant photosystem I binds light-harvesting complex I on one side of the complex. Biochemistry 40, 1029–1036.
[4] Ben-Shem, A., Frolow, F. and Nelson, N. (2003) Crystal structure of plant photosystem I. Nature 426, 630–635.
[5] Croce, R. and Bassi, R. (1998) The light-harvesting complex of photosystem I: pigment composition and stoichiometry in: Photosyntesis: Mechanisms and Effects (Garab, G., Ed.), pp. 421–424, Kluwer Academic Publishers, Dordrecht.
[6] Croce, R., Morosinotto, T., Castelletti, S., Breton, J. and Bassi, R. (2002) The Lhca antenna complexes of higher plants photosystem I. Biochim. Biophys. Acta 1556, 29–40.
[7] Bailey, S., Walters, R.G., Jansson, S. and Horton, P. (2001) Acclimation of *Arabidopsis thaliana* to the light environment: the existence of separate low light an high light responses. Planta 213, 794–801.
[8] Gobets, B. and van Grondelle, R. (2001) Energy transfer and trapping in photosystem I. Biochim. Biophys. Acta 1507, 80–99.
[9] Croce, R., Zucchelli, G., Garlaschi, F.M., Bassi, R. and Jennings, R.C. (1996) Excited state equilibration in the photosystem I light-harvesting I complex: P700 is almost isoenergetic with its antenna. Biochemistry 35, 8572–8579.
[10] Croce, R., Zucchelli, G., Garlaschi, F.M. and Jennings, R.C. (1998) A thermal broadening study of the antenna chlorophylls in PSI-200, LHCI, and PSI core. Biochemistry 37, 17355–17360.
[11] Jennings, R.C., Zucchelli, G., Croce, R. and Garlaschi, F.M. (2003) The photochemical trapping rate from red spectral states in PSI-LHCI is determined by thermal activation of energy transfer to bulk chlorophylls. Biochim. Biophys. Acta 1557, 91–98.
[12] Ratsep, M., Johnson, T.W., Chitnis, P.R. and Small, R.C. (2000) The red-absorbing chlorophyll a antenna states of photosystem I: a hole-burning study of *Synechocystis* sp. PCC 6803 and its mutants. J. Phys. Chem. B 104, 836–847.
[13] Hayes, J.M., Matsuzaki, S., Ratsep, M. and Small, G.J. (2000) Red chlorophyll a antenna states of photosystem I of the cyanobacterium *Synechocystis* sp. PCC 6803. J. Phys. Chem. B 104, 5625–5633.
[14] Engelmann, E., Tagliabue, T., Karapetyan, N.V., Garlaschi, F.M., Zucchelli, G. and Jennings, R.C. (2001) CD spectroscopy provides evidence for excitonic interactions involving red-shifted chlorophyll forms in photosystem I. FEBS Lett. 499, 112–115.
[15] Ihalainen, J.A., Ratsep, M., Jensen, P.E., Scheller, H.V., Croce, R., Bassi, R., Korppi-Tommola, J.E.I. and Freiberg, A. (2003) Red spectral forms of chlorophylls in green plant PSI – a site-selective and high-pressure spectroscopy study. J. Phys. Chem. B 107, 9086–9093.
[16] Morosinotto, T., Breton, J., Bassi, R. and Croce, R. (2003) The nature of a chlorophyll ligand in Lhca proteins determines the far red fluorescence emission typical of photosystem I. J. Biol. Chem. 278, 49223–49229.
[17] Ben-Shem, A., Frolow, F. and Nelson, N. (2004) Light-harvesting features revealed by the structure of plant photosystem I. Photosynth. Res. 81, 239–250.
[18] Knoetzel, J., Bossmann, B. and Grimme, L.H. (1998) Chlorina and viridis mutants of barley (*Hordeum vulgare* L.) allow assignment of long-wavelength chlorophyll forms to individual lhca proteins of photosystem I in vivo. FEBS Lett. 436, 339–342.
[19] Melkozernov, A.N., Lin, S., Schmid, V.R.H., Paulsen, H., Schmidt, G.W. and Blankenship, R.E. (2000) Ultrafast excitation dynamics of low energy pigments in reconstituted peripheral light-harvesting complexes of photosystem I. FEBS Lett. 471, 89–92.
[20] Schmid, V.H.R., Thome, P., Ruhle, W., Paulsen, H., Kuhlbrandt, W. and Rogl, H. (2001) Chlorophyll *b* is involved in long-wavelength spectral properties of light-harvesting complexes LHC I and LHC II. FEBS Lett. 499, 27–31.
[21] Castelletti, S., Morosinotto, T., Robert, B., Caffarri, S., Bassi, R. and Croce, R. (2003) Recombinant Lhca2 and Lhca3 subunits of the photosystem I antenna system. Biochemistry 42, 4226–4234.
[22] Rivadossi, A., Zucchelli, G., Garlaschi, F.M. and Jennings, R.C. (1999) The importance of PSI chlorophyll red forms in light-harvesting by leaves. Photosynth. Res. 60, 209–215.
[23] Cometta, A., Zucchelli, G., Karapetyan, N.V., Engelmann, E., Garlaschi, F.M. and Jennings, R.C. (2000) Biophys. J. 79, 3235–3243.
[24] Ihalainen, J.A., Gobets, B., Sznee, K., Brazzoli, M., Croce, R., Bassi, R., van Grondelle, R., Korppi-Tommola, J.E.I. and Dekker, J.P. (2000) Evidence for two spectroscopically different dimers of light-harvesting complex I from green plants. Biochemistry 39, 8625–8631.
[25] Jennings, R.C., Garlaschi, F.M., Morosinotto, T., Engelmann, E. and Zucchelli, G. (2003) The room temperature emission band shape of the lowest energy chlorophyll spectral form of LHCI. FEBS Lett. 547, 107–110.
[26] Jennings, R.C., Zucchelli, G., Engelmann, E. and Garlaschi, F.M. (2004) The long wavelength chlorophyll state of plant LHCI at room temperature: a comparison with PSI-LHCI. Biophys. J. 87, 488–497.
[27] Zucchelli, G., Jennings, R.C., Garlaschi, F.M., Cinque, G., Bassi, R. and Cremonesi, O. (2002) The calculated in vitro and in vivo chlorophyll a absorption bandshape. Biophys. J. 82, 378–390.
[28] Schmid, V.H.R., Cammarata, K.V., Bruns, B.U. and Schmidt, G.W. (1997) In vitro reconstitution of the photosystem I light-harvesting complex Lhci-730: heterodimerization is required for antenna pigment organisation. Proc. Natl. Acad. USA 94, 7667–7672.
[29] Nagai, K. and Thøgersen, H.C. (1987) Synthesis and sequence-specific proteolysis of hybrid proteins produced in Escherichia coli. Methods Enzymol. 153, 461–481.
[30] Paulsen, H., Finkenzeller, B. and Kuhlein, N. (1993) Pigment induce folding of light-harvesting chlorophyll alpha/beta-binding protein. Eur. J. Biochem. 215, 809–816.
[31] Giuffra, E., Cugini, D., Croce, R. and Bassi, R. (1996) Reconstitution and pigment-binding properties of recombinant CP29. Eur. J. Biochem. 238, 112–120.
[32] Gilmore, A.M. and Yamamoto, H.Y. (1991) Zeaxanthin formation and energy-dependent fluorescence quenching in pea chloroplasts under artificially mediated linear and cyclic electron transport. Plant Physiol. 96, 635–643.

[33] Croce, R., Canino, G., Ros, F. and Bassi, R. (2002) Chromophore organization in the higher-plant photosystem II antenna protein CP26. Biochemistry 41, 7334–7343.
[34] Lakowicz, J.R. (1999) Principles of Fluorescence Spectroscopy, Kluwer/Plenum Press, New York.
[35] Hilbert, M., Wehling, A., Schlodder, E. and Walla, P.J. (2004) Two-photon-sensitized fluorescence and excitation spectra of photosystem I of *Thermosynechococcus elongatus*. J. Phys. Chem. B. 108, 13022–13030.
[36] Gobets, B., Kennis, J.T.M., Ihalainen, J.A., Brazzoli, M., Croce, R., van Stokkum, I.H.M., Bassi, R., Dekker, J.P., van Amerongen, H., Fleming, G.R. and van Grondelle, R. (2001) Excitation energy transfer in dimeric light-harvesting complex I: a combined streak camera/fluorescence upconversion study. J. Phys. Chem. B 105, 4485–4494.
[37] Zucchelli, G., Garlaschi, F.M. and Jennings, R.C. (1996) Thermal broadening analysis of the light harvesting complex II absorption spectrum. Biochemistry 35, 16247–16254.
[38] Croce, R., Morosinotto, T., Ihalainen, J.A., Chojnicka, A., Breton, J., Dekker, J.P., van Grondelle, R. and Bassi, R. (2004) Origin of the 701-nm fluorescence emission of the Lhca2 subunit of higher plant photosystem I. J. Biol. Chem. 279, 48543–48549.
[39] Berman, H.M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T.N., Weissig, H., Shindyalov, I.N. and Bourne, P.E. (2000) The protein data bank. Nucleic Acid Res. 28, 235–242.
[40] Jordan, P., Fromme, P., Witt, H.T., Klukas, O., Saenger, W. and Krauss, N. (2001) Three-dimensional structure of cyanobacterial photosystem I at 2.4 Angstrom resolution. Nature 411, 909–917.